Dermal Fibroblasts Promote the Migration of Dendritic Cells

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Migration of dendritic cells (DCs) from skin to lymph nodes on activation is an essential step in the initiation of an adequate immune response. The dermal microenvironment including stromal cells and their soluble factors might be involved in the regulation of DC migration. To focus on the role of dermal fibroblasts, we studied whether interaction of DCs with fibroblasts promotes the migration of DCs. DCs were co-cultured with resting fibroblasts or with tumor necrosis factor (TNF)α/IL-1β-activated fibroblasts to mimic an inflammatory microenvironment. Interaction of DCs with TNFα/IL-1β-stimulated fibroblasts increased the secretion of matrix metalloproteinase-9 (MMP-9) from DCs within 6 hours compared with DCs alone or DCs stimulated with lipopolysaccharide or TNFα/IL-1β. In contrast, unstimulated fibroblasts did not affect MMP-9 secretion. IL-6 released by TNFα/IL-1β-stimulated fibroblasts was identified as a factor responsible for fibroblast-stimulated MMP-9 secretion from DCs. In accordance with the elevated MMP-9 release, on co-culture with TNFα/IL-1β-stimulated fibroblasts, DCs migrated significantly more effectively through matrigel matrices than did TNFα/IL-1β-stimulated DCs. This was inhibited by a selective blocking of MMP-9, indicating the importance of MMP-9 for this migratory capacity of DCs. In summary, fibroblasts in the local dermal microenvironment are capable of potentiating the migratory capacity of DCs, and thus have the potential to actively participate in the regulation of a cutaneous immune response.

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INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells found in an immature stage in epithelia and in the interstitial space of most solid organs, where they capture antigens such as invading bacteria, viruses, or damaged tissue. On receipt of the so-called ''danger signals'' or in response to proinflammatory cytokines or T-cell-derived signals such as CD40 ligand (CD40 L), DCs mature and also undergo loss of phagocytic capacity and upregulation of costimulatory molecules, major histocompatibility complex class II, and cytokines (Steinman, 2007). Most importantly, DCs acquire the ability to migrate from peripheral tissues to the T-cell areas of draining lymphoid organs. This is associated with the expression of chemokine receptors resulting in a responsiveness to CCL21 and CCL19, and the expression of matrix-degrading enzymes such as matrix metalloproteinases (MMP) (Banchereau and Steinman, 1998); (Steinman *et al.*, 1995). Once in lymph nodes, they initiate immune responses by activating T cells and controlling the differentiation of T cells either to a TH1, TH2, or TH17 phenotype or to regulatory T cells (Steinman, 2007).

In this process, DCs have to move through connective tissues and cross basement membranes. MMP-9 is especially important in migration, as it cleaves collagen IV, the major component of basement membranes. The role of MMP-9 in DC migration has been demonstrated *in vitro,* in skin explant models, and *in vivo* after treatment with anti-MMP-9 antibodies, as well as in MMP-9-deficient mice (Yen *et al.*, 2007). Thus, DCs from MMP-9-deficient mice exhibit a significantly reduced emigration of Langerhans cells (LCs) from skin explant culture models (Ratzinger *et al.*, 2002).

In addition to its matrix-degrading capacity, MMP-9 is also involved in the processing of cytokines and chemokines relevant for inflammation and migration (Opdenakker *et al.*, 2001a, 2001b). Furthermore, MMP-9 supported the migration of DCs independent of an extracellular barrier (Hu and Ivashkiv, 2006). Hu and Ivashkiv (2006) showed that MMP-9 enhanced the CCL5-mediated signaling by a synergistic activation of JNK. Thus, MMP-9 may have an effect as a tuner and amplifier of immune functions by cleavage of matrix components and modification of cytokine and chemokine activity (Opdenakker *et al.*, 2001b).

It was demonstrated in recent years that the stromal microenvironment is actively involved in the regulation of

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Abbreviations: IL-1β, interleukin-1β; IL-6, interleukin-6; LC, Langerhans cell; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; TLR, toll-like receptor; TNFα, tumor necrosis factor α

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immune responses (Svensson and Kaye, 2006). Zhang *et al.* (1998) described that splenic stromal cells drive DCs to differentiate into a regulatory phenotype. Hyaluronic acid and osteopontin regulate the differentiation and maturation of DCs (Termeer *et al.*, 2003; Renkl *et al.*, 2005). Fibroblasts in particular display a wide and varied biosynthetic repertoire, including the production of matrix components, matrix-degrading enzymes, cytokines, chemokines, and prostanoids (Parsonage *et al.*, 2003; Buckley *et al.*, 2001, 2004). Thus, it is likely that DCs receive information from the local environment. For example, the lack of matrix-associated SPARC (secreted protein, acidic and rich in cysteine) in skin increased the emigration of LCs from the skin to the lymph node. In this process, a cutaneous contact hypersensitivity reaction was enhanced (Sangaletti *et al.*, 2005). Moreover, the local microenvironment is involved in the regulation of the organ-specific homing of T cells. Thus, subcutaneous but not intraperitoneal application of DCs stimulated the expression of skin-homing molecules on T cells, resulting in an enhanced cutaneous contact hypersensitivity reaction (Dudda *et al.*, 2004). Recently, we showed that Thy-1 expressed in fibroblasts and activated endothelial cells stimulated the secretion of MMP-9 from neutrophils, resulting in an enhanced migration through extracellular matrix (Saalbach *et al.*, 2008). Thus, all the data support the fact that immunocompetent cells receive functionally important information from the local environment.

In this study, we demonstrate that activated fibroblasts provided a microenvironment that directly and rapidly enhanced the migratory capacity of DCs by stimulating their MMP-9 secretion. IL-6 released from fibroblasts was identified as an essential mediator in this process, in which fibroblasts actively participate in the regulation of an immune response.

RESULTS

Activated human dermal fibroblasts stimulate the secretion of MMP-9 from DCs

Upon activation, DCs mature and acquire the ability to migrate through the extracellular matrix from peripheral tissues to the T-cell areas of draining lymphoid organs. As MMP-9 is important for the migration of DCs, we were interested to determine whether dermal fibroblasts as cells of the dermal microenvironment were able to regulate the expression and secretion of MMP-9 from DCs. Fibroblasts were co-cultured with immature DCs for 6 hours to study their role in the regulation of MMP-9 release from DCs and their contribution to DC migration. DCs were cultured without stimulus as control or were stimulated with tumor necrosis factor (TNF)α/IL-1β or lipopolysaccharide (LPS). As DC migration is required during steady-state conditions in the absence of an inflammation, as well as during an inflammatory reaction (Steinman, 2007), we used resting fibroblasts and fibroblasts stimulated with TNFα and IL-1β for 18 hours to mimic an inflammatory microenvironment. TNFα and IL-1β were chosen because they are highly expressed in cutaneous inflammation (Taylor *et al.*, 2004; Aggarwal *et al.*, 2006).

Immature DCs expressed only small amounts of MMP-9. TNFα/IL-1β significantly stimulated MMP-9 secretion from immature DCs. Interestingly, the initiation of DC maturation by adding LPS did not significantly stimulate MMP-9 secretion within 6 hours (Figure 1a, b). Unstimulated fibroblasts did not enhance MMP-9 secretion from immature DCs.

Importantly, in the co-culture of DCs with TNFα/IL-1β-stimulated fibroblasts, the mRNA expression and secretion of MMP-9 in DCs were significantly increased compared with that in immature DCs, as well as in DC stimulated with LPS or TNFα/IL-1β (Figure 1a–c). Although the addition of TNFα and IL-1β stimulated MMP-9 mRNA expression and secretion to DCs alone, DCs in the co-culture with fibroblasts activated by the same concentration of TNFα and IL-1β secreted significantly more MMP-9 (Figure 1b and c).

As cell-free supernatants of TNFα/IL-1β-activated fibroblasts also stimulated MMP-9 secretion from immature DCs in the same manner as did the direct co-culture, we postulated that fibroblast-derived soluble mediators are responsible for MMP-9 upregulation in DCs. Second, we could assess that MMP-9 in the co-culture was derived from DCs, as fibroblasts did not express MMP-9 (Figure 1b).

Next, we were interested in determining whether the stimulation of MMP-9 secretion by activated fibroblasts is dependent on the maturational state of DCs. Thus, we compared immature DCs and fully matured DCs. Furthermore, in an attempt to imitate the situation of DCs at first contact with pathogens and the subsequent initiation of DC maturation, we preincubated DCs with LPS for 1 or 3 hours (partially matured DCs).

Immature DCs, partially matured DCs, and LPS-matured DCs were stimulated with supernatants of TNFα/IL-1β-stimulated fibroblasts. In another panel, DCs were stimulated simultaneously with supernatants of activated fibroblasts and LPS. As shown in Figure 1d, soluble factors of activated fibroblasts increased the secretion of MMP-9 from both immature and partially matured DCs, and from DCs stimulated simultanously with LPS and supernatants of activated fibroblasts. In contrast, MMP-9 secretion from mature DCs was not affected by soluble mediators from activated fibroblasts.

To test whether results obtained with monocyte-derived DCs are of general importance for DCs traveling from skin to lymph nodes, we performed additional experiments using LCs. LCs were generated *in vitro* by a differentiation of monocytes in the presence of IL-4, GM-CSF, and TGFβ. In addition, LCs were isolated from human epidermis. Of the isolated LCs, 80–90% expressed Langerin, confirming the LC phenotype (data not shown). LCs were cultured with supernatants of TNFα/IL-1β-stimulated fibroblasts or with TNFα/IL-1β as control. As shown in Figure 1e, soluble factors of TNFα/IL-1β-stimulated fibroblasts enhanced MMP-9 secretion from both *in vitro*-generated LCs as well as from LCs isolated from human epidermis.

Induction of DC maturation through toll-like receptors is not mandatorily associated with the upregulation of MMP-9 secretion

In previous experiments, we observed that stimulation of DCs with LPS that signals through toll-like receptor 4 (TLR4) did not stimulate MMP-9 secretion. On the other hand, LPS is a

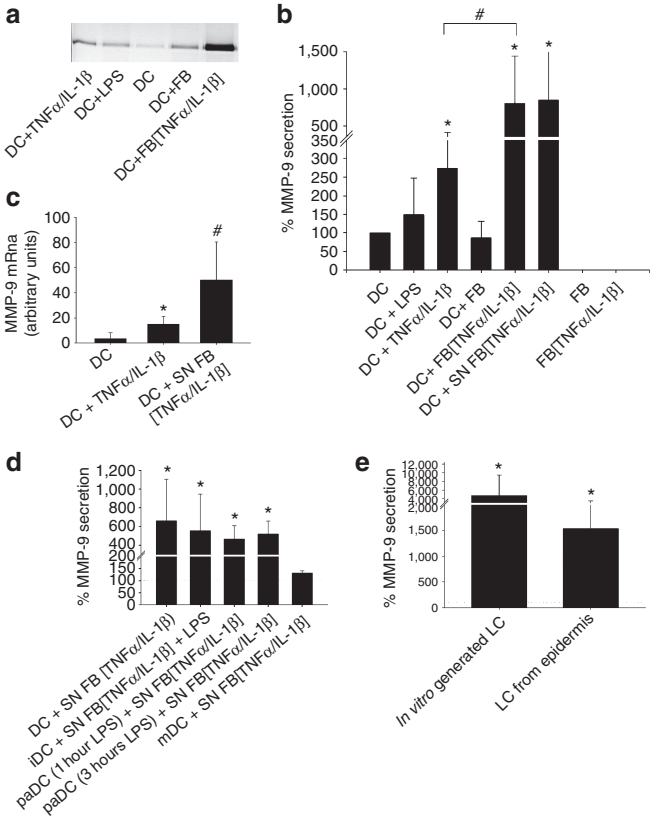

Figure 1. Tumor necrosis factor (TNF)α/IL-1β-treated human fibroblasts stimulate matrix metalloproteinase-9 (MMP-9) secretion by dendritic cells (DCs). (**a**) Monocyte-derived immature DCs were co-cultured with human dermal fibroblasts or with TNFα/IL-1β-stimulated fibroblasts (FB[TNFα/IL-1β]) for 6 hours. As control, DCs were cultured with 1 μg ml^{-1} LPS, 5 ng ml^{-1} TNFα/2.5 ng ml^{-1} IL-1β, or without stimulus. One representative zymogram is shown (**b**). Immature DCs were co-cultured with fibroblasts (FB), with TNFα/IL-1β-stimulated fibroblasts (FB[TNFα/IL-1β]), or with supernatants (SN) of TNFα/IL-1β-activated FB for 6 hours. In parallel, DCs were cultured with 1 μg ml^{-1} LPS; 5 ng ml^{-1} TNFα/2.5 ng ml^{-1} IL-1β; or without stimulus. Band intensity in gelatine zymography was analyzed by densitometric evaluation. Unstimulated DCs were used as control and were set to 100%. The mean ± SD of five independent experiments is shown. *P<0.05 compared with DCs; $^{\#}P$<0.05 compared with DCs + TNFα/IL-1β (Mann–Whitney rank-sum test). (**c**) DCs were stimulated with supernatants of TNFα/IL-1β-stimulated fibroblasts (SN FB[TNFα/IL-1β]). As control, DCs were cultured with recombinant TNFα/IL-1β or without stimulus. After 6 hours, RNA was prepared and MMP-9 mRNA expression was detected by quantitative real time–PCR. The mean ± SD of three independent experiments is shown. *P<0.05 compared with DCs; $^{\#}P$<0.05 compared with TNFα/IL-1β-stimulated DC (Mann–Whitney rank-sum test). (**d**) Immature DCs (iDC), partially mature (1 hour or 3 hours LPS maturation before stimulation, paDC) DCs or mature DCs (mDC) were stimulated with supernatants of TNFα/IL-1β-stimulated fibroblast. In addition, fibroblasts were stimulated simultaneously with supernatants of TNFα/IL-1β-stimulated fibroblasts and LPS. MMP-9 secretion was detected by gelatine zymography. Band intensity was analyzed by densitometric evaluation. As control, DCs stimulated with TNFα/IL-1β with or without LPS were used and were set to 100%. The mean ± SD of three independent experiments is shown. *P<0.05 compared with DCs + TNFα/IL-1β (Mann–Whitney rank-sum test). (**e**) *In vitro* generated LCs or LCs isolated from human epidermis were incubated with supernatants of TNFα/IL-1β-stimulated fibroblasts or TNFα/IL-1β as control. MMP-9 secretion was detected by gelatine zymography. Band intensity was analyzed by densitometric evaluation. LC stimulated with TNFα/IL-1β were used as control and were set to 100%. The mean ± SD of three independent experiments is shown. *P<0.05 compared with DCs + TNFα/IL-1β (Mann–Whitney rank-sum test).

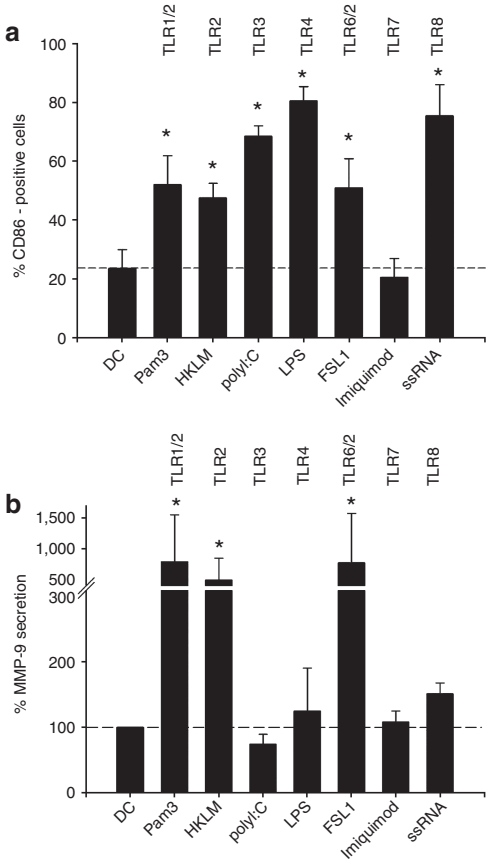

Figure 2. TLR3-, TLR4, and TLR8 agonists induce maturation of dendritic cells (DCs), but do not stimulate their MMP-9 secretion. Immature DCs were stimulated with Pam3 (synthetic triacetylated lipoprotein; 0.5 μg ml^{-1}), HKLM (heat-killed preparation of Listeria monocytogenes, 10^6 cells ml^{-1}), polyI:C (synthetic analog of double-stranded RNA; 10 μg ml^{-1}), LPS (0.5 μg ml^{-1}), FSL1 (synthetic diacetylated lipoprotein; 0.5 μg ml^{-1}), Imiquimod (1 μg ml^{-1}), and ssRNA (single-stranded RNA 40; 1 μg ml^{-1}). The TLR that was stimulated by the appropriate agonist was noted. (**a**) DCs were stimulated for 24 hours. CD86 expression was evaluated by flow cytometry. (**b**) Supernatants were collected after 6 hours and MMP-9 secretion was detected by gelatine zymography. Band intensity was detected by densitometric evaluation. Unstimulated DCs were used as control and was set to 100%. The mean ± SD of three independent experiments is shown. *P<0.05 compared with that of DCs (*t*-test).

potent inducer of DC maturation. Now, we were interested in determining whether other pathogen-associated ligands recognized by TLR could regulate the secretion of MMP-9. Iwasaki and Medzhitov (2004) described the expression of TLR1–4, TLR6, and TLR8 on *in vitro*-generated monocyte-derived DCs. Therefore, we stimulated DCs with the corresponding agonists. Stimulation of TLR by the indicated agonists induced the maturation of DCs, which was shown by an increased expression of costimulatory molecules (CD80, CD83, and CD86) and MHC II. In Figure 2a, the expression of CD86 is exemplarily shown. As monocyte-derived DCs did not express TLR7, Imiquimod, a TLR7 agonist, was used as negative control. Consequently, Imiquimod did not induce the maturation of DCs. In spite of the induction of DC maturation by TLR3-, TLR4-, and TLR8 activation, these

agonists did not enhance the MMP-9 secretion detected by gelatine zymography after 6 hours of stimulation (Figure 2b). In contrast, the stimulation of DCs with agonists of TLR1/2 (Pam3) or TLR6/2 (FSL1) induced the maturation of DCs as well as the stimulation of MMP-9 release. Thus, stimulation of DCs through TLR induced the maturation of DCs, but this is not necessarily associated with an upregulation of MMP-9 secretion.

Taken together, our data support the notion that, in addition to ''classical'' DC maturation signals as mediated by TLRs, supplementary signals from the tissue microenvironment are required to induce a rapid release of MMP-9. In this manner, the stromal fibroblast from the dermal microenvironment might facilitate DC migration.

Fibroblast-derived IL-6 is involved in the stimulation of MMP-9 secretion from DCs on contact with fibroblasts

Next, we studied the mechanisms of fibroblast-stimulated MMP-9 secretion. We speculated that fibroblast-derived soluble factors were mainly responsible for upregulated MMP-9 secretion, because cell-free supernatants of activated fibroblasts stimulated MMP-9 secretion in a similar manner as did the direct co-culture (Figure 1). As fibroblasts produce IL-6 and prostaglandin E2 (PGE2) upon activation through CD40, we verified the release of these mediators after stimulation with TNFα/IL-1β (Smith *et al.*, 1997a, 1997b; Parsonage *et al.*, 2003; Buckley *et al.*, 2001, 2004). TNFα/IL-1β-stimulated fibroblasts produced $3300 \pm 2300\,pg\,ml^{-1}$ of IL-6 and $3200 \pm 1400\,pg\,ml^{-1}$ PGE2 (Figure 3a). To identify which of these mediators were responsible for the increase of MMP-9 secretion, DCs were stimulated for 6 hours with $0.1–10\,ng\,ml^{-1}$ recombinant IL-6 or $3.5\,ng\,ml^{-1}$ PGE2. In addition, we used $352\,ng\,ml^{-1}$ (10^{-6} M) PGE2, as this concentration was reported to induce MMP-9 secretion from DCs (Yen *et al.*, 2007). As shown in Figure 3b, IL-6 was a potent inducer of MMP-9 secretion from DCs in the concentration range found in supernatants of activated fibroblasts. In contrast, PGE2 stimulated MMP-9 secretion only at high concentrations ($352\,ng\,ml^{-1}$ 10^{-6} M). At a lower concentration ($3.5\,ng\,ml^{-1}$), as found in our co-cultures, PGE2 was not able to increase MMP-9 secretion from DCs.

As IL-6 was a potent inducer of MMP-9 in DCs, we studied the role of IL-6 in the fibroblast-mediated stimulation of MMP-9 secretion of DCs. Secretion of IL-6 by fibroblasts was inhibited by small interfering RNA (siRNA) silencing. Fibroblasts were transfected with IL-6 siRNA or as control with scrambled siRNA. After transfection, fibroblasts were stimulated with TNFα/IL-1β for 24 hours to induce IL-6 secretion. Efficiency of IL-6 silencing was determined by the detection of IL-6 in supernatant using ELISA. We used two separate IL-6 siRNAs. Both downregulated the IL-6 expression (data not shown). The most effective siRNA was used in subsequent experiments. As shown in Figure 4a and b, silencing of IL-6 resulted in a significant decrease but not in a complete inhibition of IL-6 secretion. Transfection with scrambled siRNA did not significantly affect IL-6 secretion compared with nontransfected fibroblasts.

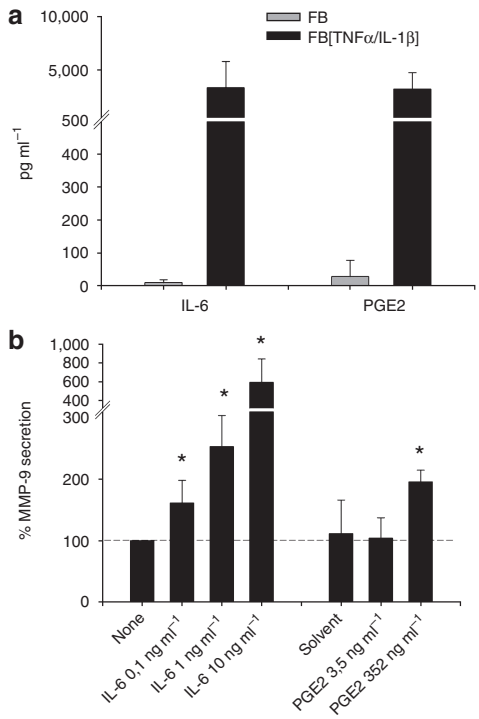

Figure 3. IL-6 induces the secretion of matrix metalloproteinase-9 (MMP-9) from dendritic cells (DCs). (**a**) Fibroblasts were cultured for 18 hours with $5\,ng\,ml^{-1}$ tumor necrosis factor-α (TNFα) and $2.5\,ng\,ml^{-1}$ IL-1β, or in serum-free medium. IL-6 and prostaglandin E2 (PGE2) were measured in the supernatant by ELISA. (**b**) DCs were stimulated with indicated concentrations of IL-6 or PGE2 for 6 hours. As control, DCs were cultured without stimulation. MMP-9 was detected in the supernatant by gelatine zymography. Band intensity was detected by densitometric evaluation. MMP-9 secretion from unstimulated DCs was set to 100%. The mean ± SD of three independent experiments is shown. *$P<0.05$ compared with that of DCs (*t*-test).

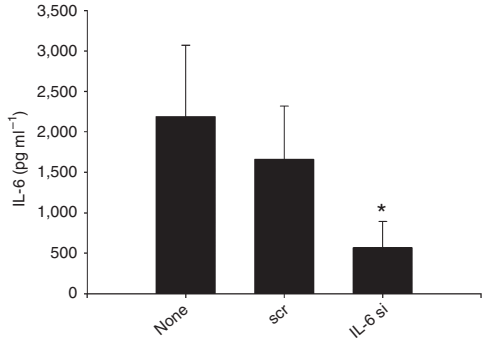

Figure 4. Small interfering RNA (siRNA) silencing of IL-6 resulted in downregulation of IL-6 from activated fibroblasts. Fibroblasts were transfected with IL-6 siRNA or scrambled (scr) siRNA. As control, untreated fibroblasts were used. After 24 hours, transfected fibroblasts were stimulated with tumor necrosis factor-α (TNFα)/IL-1β. IL-6 was detected in the supernatants after 24 hours by ELISA. The mean ± SD of five independent experiments is shown.*$P<0.05$ compared with that of dendritic cells (*t*-test).

Next, we tested whether IL-6 is the relevant fibroblast-derived mediator that induces MMP-9 secretion from DCs. To address this issue, DCs were stimulated with supernatants of fibroblasts transfected with IL-6 siRNA or scrambled siRNA.

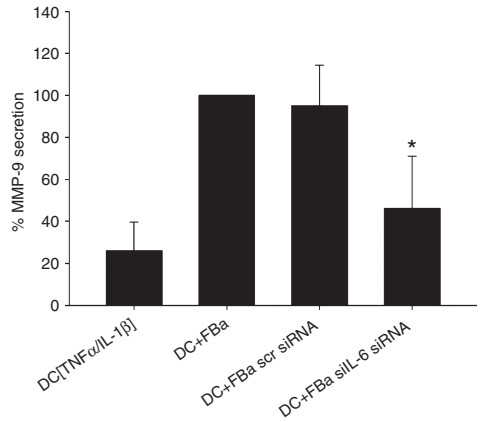

Figure 5. The knockdown of IL-6 expression by siRNA silencing in fibroblasts results in a significant decrease in fibroblast-stimulated MMP-9 secretion from DCs. DCs were stimulated with supernatants of tumor necrosis factor-α (TNFα)/IL-1β-stimulated fibroblasts transfected with IL-6 siRNA or scrambled (scr) siRNA, respectively. As control, DCs were incubated with TNFα/IL-1β or with supernatants of TNFα/IL-1β-stimulated fibroblasts (FBa). Supernatants were collected after 6 hours and MMP-9 release was detected by gelatine zymography. Band intensity was analyzed by densitometric evaluation. MMP-9 secretion of DCs stimulated with supernatant from TNFα/IL-1β-stimulated fibroblasts was set to 100%. Data represent the mean ± SD of five independent experiments. $*P<0.05$ compared with that of DCs + FBa (Mann–Whitney rank-sum test).

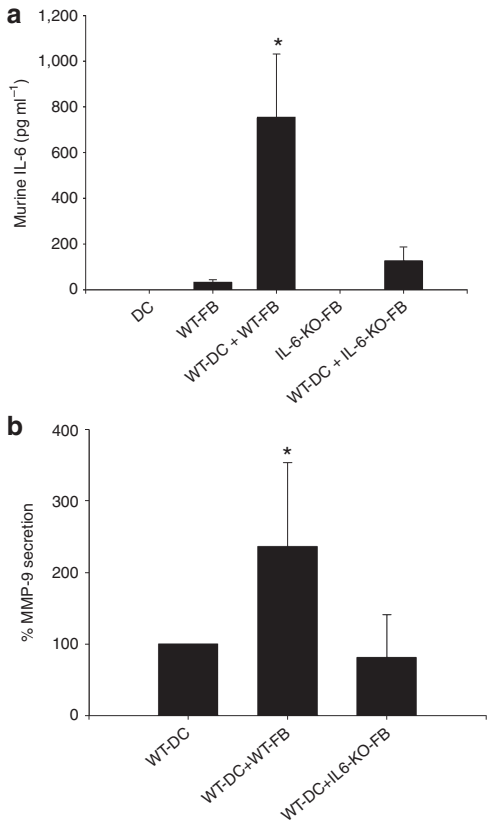

Figure 6. Murine fibroblasts stimulate MMP-9 secretion from murine bone marrow-derived dendritic cell (DC) through IL-6 secretion. Murine bone marrow-derived DCs (WT-DCs) from BALB/c mice were co-cultured with fibroblasts from IL-$6^{-/-}$ mice (IL-6-KO-FB) or from wild-type controls (WT-FB). As control, DCs or fibroblasts were cultured alone. Supernatants were collected after 6 hours. (**a**) Murine IL-6 was detected by ELISA. $*P<0.05$ compared with that of WT-FB and WT-DCs + IL-6-KO-FB (Mann–Whitney rank-sum test). (**b**) MMP-9 secretion was detected by gelatine zymography. Band intensity was analyzed by densitometric evaluation. Data represent the mean ± SD of five independent experiments. $*P<0.05$ compared with that of WT-DCs and WT-DCs + IL-6-KO-FB (Mann–Whitney rank-sum test).

As control, DCs were stimulated either with TNFα/IL-1β or with supernatants of TNFα/IL-1β-activated fibroblasts. Again, the incubation of DCs with supernatants of TNFα/IL-1β-activated fibroblasts clearly enhanced MMP-9 secretion from DCs compared with DCs stimulated with TNFα/IL-1β (Figure 5). It is noteworthy that the inhibition of IL-6 secretion from TNFα/IL-1β-activated fibroblasts resulted in a significant decrease in MMP-9 release from DCs. Transfection with scrambled siRNA did not affect MMP-9 secretion (Figure 5).

To confirm these results in another experimental system, murine DCs were co-cultured with fibroblasts from IL-6-deficient mice or wild-type mice. Wild-type fibroblasts secreted low levels of IL-6 ($32 \pm 10\,pg\,ml^{-1}$). In contrast to human unstimulated fibroblasts, IL-6 was already clearly upregulated in the co-culture of DCs and unstimulated wild-type fibroblasts ($754 \pm 254\,pg\,ml^{-1}$). In contrast, fibroblasts from IL-6-deficient mice did not produce IL-6. In the co-culture of IL-6-deficient fibroblasts with DCs, only small amounts of IL-6 were detectable ($109 \pm 54\,pg\,ml^{-1}$) (Figure 6a). Consequently, fibroblasts from IL-$6^{-/-}$ mice stimulated MMP-9 secretion in DCs to a significantly smaller extent than did fibroblasts from wild-type controls (Figure 6b).

Taken together, our data underline the fact that fibroblast-derived IL-6 was involved in the regulation of fibroblast-stimulated MMP-9 secretion from DCs.

Activated human dermal fibroblasts stimulate the migratory capacity of DCs depending on MMP-9

As MMP-9 is important for the migration of DCs through basement membranes, we studied the functional relevance of fibroblast-stimulated MMP-9 secretion in DCs in migration assays through matrigel, a basement membrane-like structure. DCs were incubated with activated fibroblasts for 2 hours. As control, DCs stimulated with TNFα/IL-1β were used. DCs (5×10^5) were seeded in the upper chamber of matrigel inserts and a medium with 1% serum was used as a chemoattractant in the lower chamber. Migrated cells were counted by flow cytometry after 24 hours. In line with their enhanced MMP-9 secretion, fibroblast-stimulated DCs migrated significantly more than TNFα/IL-1β-stimulated DCs (Figure 7a). To investigate whether fibroblast-stimulated MMP-9 secretion by DCs is involved in enhanced DC transmigration, MMP-9 activity was blocked. As shown in Figure 7b, the addition of function-blocking anti-MMP-9 antibodies significantly inhibited the fibroblast-stimulated transmigration of DCs.

In addition, DCs were stimulated with supernatants of TNFα/IL-1β-stimulated fibroblasts in which IL-6 secretion had been inhibited by siRNA silencing. As a result of IL-6 silencing in fibroblasts, MMP-9 secretion (Figure 5) and the

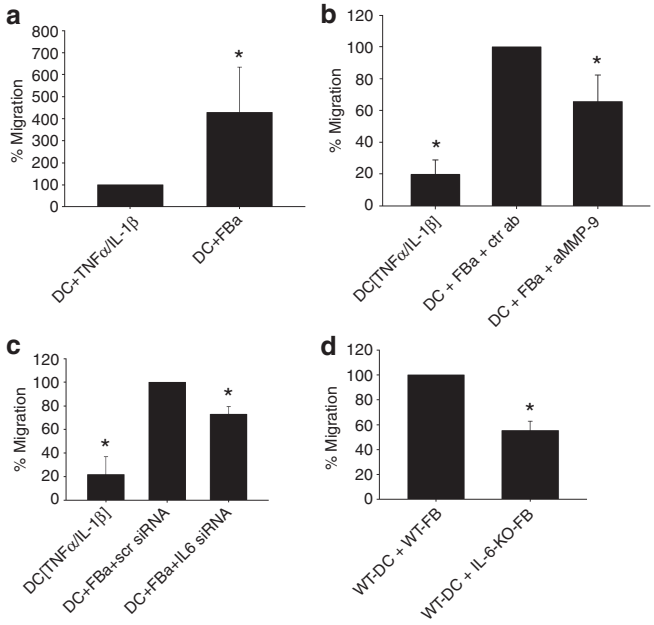

Figure 7. Tumor necrosis factor-α (TNFα)/IL-1β-stimulated fibroblasts enhanced the migration of dendritic cells (DCs) through matrigel. DCs were co-cultured with TNFα/IL-1β-stimulated fibroblasts (FBa) for 2 hours. Subsequently, DC migration through matrigel inserts was measured after 24 hours. Migrated cells were counted by flow cytometry. Data represent the mean ± SD of at least four independent experiments. $*P<0.05$ (Mann–Whitney rank-sum test). (**a**) Migration of DCs stimulated with 5 ng ml^{-1} TNFα/2.5 ng ml^{-1} IL-1β and DCs on co-culture with TNFα/IL-1β-stimulated fibroblasts (FBa) was detected. Migration of TNFα/IL-1β-stimulated DCs was set to 100%. (**b**) DCs on co-culture with TNFα/IL-1β-stimulated fibroblasts (FBa) were added to the upper part of the transmigration chamber in the presence of an anti-MMP-9 antibody or an isotype control antibody (ctr ab). DCs stimulated with TNFα/IL-1β were used as control. Migration in the presence of the ctr ab was set to 100%. (**c**) Migration of DCs on culture with supernatants of TNFα/IL-1β-stimulated fibroblasts (FBa) after IL-6 small interfering RNA (siRNA) or scrambled (scr) siRNA transfection was detected. DCs stimulated with TNFα/IL-1β were used as control. Migration on stimulation with scr-transfected fibroblast supernatants was set to 100%. (**d**) DCs from wild-type mice (WT-DCs) were collected after co-culture with wild-type fibroblasts (WT-FB) or with fibroblasts from IL-6-deficient mice (IL-6-KO-FB) and were added to the upper chamber of matrigel transwell inserts. Migration on co-culture with WT-FB was set to 100%. Data represent the mean ± SD of at least three independent experiments. $*P<0.05$ (Mann–Whitney rank-sum test).

migratory capacity of DCs were reduced (Figure 7c). Similarly, MMP-9 secretion of murine DCs on co-culture with fibroblasts from IL-6-deficient mice compared with that on co-culture with wild-type fibroblasts also resulted in a decreased DC migration through the matrigel (Figure 7d).

DISCUSSION

In this study, we demonstrate how stromal fibroblasts from the microenvironment of the skin support the migration of DCs and thus have the potential to orchestrate immune responses in skin.

Dendritic cell migration is regulated by many factors. For example, the proinflammatory cytokines, TNFα and IL-1β, can initiate the migration of DCs (van Helden *et al.*, 2006). Chemokines such as CXCL12 (SDF-1), CCL-20, CCL19, and CCL21 were shown to enhance the emigration of LCs and dermal DCs from skin (Adema *et al.*, 2005; Ouwehand *et al.*, 2008). Interactions with the extracellular matrix through CD44 or integrins are important for the migration of DCs (Price *et al.*, 1997; Weiss *et al.*, 1997). Finally, proteases degrade the extracellular matrix and thus are critically involved in the regulation of cell migration. Specifically, Ratzinger *et al.* (2002) demonstrated that MMP-2 and MMP-9 are essential for the emigration of LCs from epidermal skin explants, as well as for the emigration of dermal DCs from the dermis. The importance of MMP-9 in the emigration of DCs from epidermis and dermis was shown by function-blocking antibodies, MMP inhibitors, and MMP-9-deficient mice (Ratzinger *et al.*, 2002; Yen *et al.*, 2007).

Stromal cells are connective tissue cells forming and organizing the local stromal microenvironment. They have important functions in tissue development and homeostasis such as in coordinating matrix remodeling, in the deposition of ECM components, in tissue scaffolding, and in the regulation of cellular migration (Svensson and Kaye, 2006). Fibroblasts display a wide and variable biosynthetic repertoire, including the production of matrix components, matrix-degrading enzymes, cytokines, chemokines, and prostanoids (Buckley *et al.*, 2001, 2004; Parsonage *et al.*, 2003, 2004).

On the basis of these data, we were interested in determining whether fibroblasts regulate the migration of DCs through the stimulation of MMP-9 secretion. As DC migration is essential during both steady-state conditions in the absence of inflammation, as well as during inflammation, DCs were co-cultured either with resting fibroblasts or with fibroblasts stimulated with TNFα and IL-1β to mimic a proinflammatory tissue microenvironment. TNFα and IL-1β were chosen as stimuli, as they are highly expressed during cutaneous inflammation (Taylor *et al.*, 2004; Aggarwal *et al.*, 2006). Resting fibroblasts did not significantly enhance the secretion of MMP-9 from immature DCs. In contrast, fibroblasts stimulated by TNFα and IL-1β clearly increased the secretion of MMP-9 from DCs. Specifically, MMP-9 transcription was induced in DCs on interaction with TNFα/IL-1β-stimulated fibroblasts, as shown by quantitative real-time–PCR. To exclude that this effect was merely due to TNFα/IL-1β, DCs were stimulated with the same concentrations of TNFα/IL-1β in the absence of fibroblasts. It is noteworthy that co-culture of DCs with TNFα/IL-1β-stimulated fibroblasts significantly enhanced MMP-9 secretion up to 10-fold more than that with TNFα/IL-1β alone. As cell-free supernatants of TNFα/IL-1β-treated fibroblasts stimulated MMP-9 secretion in a manner similar to the direct DCs–fibroblast interaction, soluble fibroblast-derived factors had to be responsible.

Several reports described the importance of MMP-9 in the migration of DCs (Ratzinger *et al.*, 2002; Yen *et al.*, 2007). Indeed, we demonstrate in this study that enhanced MMP-9 secretion of fibroblast-stimulated DCs resulted in an increased transmigration through basement membrane-like structures. A decrease in this enhanced transmigration by anti-MMP-9 antibodies proved the important role of MMP-9

in the stimulation of DC migration by fibroblasts. However, MMP-9 antibodies did not completely block migration, which may be because of residual MMP-9 or a contribution of other yet unidentified MMPs. However, MMP-2 was not secreted by DCs (data not shown). In aggregate, our data demonstrate that MMP-9 released from DCs that had been activated by TNFα/IL-1β-stimulated fibroblasts is at least in part responsible for the enhanced migratory potential of fibroblast-stimulated DCs.

To study the potential role of fibroblast-stimulated MMP-9 secretion in DCs during the initial phases of infection/inflammation, we attempted to mimic the situation when DCs first encounter antigens or pathogens and the subsequent initiation of DC maturation and migration to lymph vessels. A partial maturation was induced in DCs by 1–3 hours of preincubation with LPS. Fibroblast-derived soluble factors stimulated MMP-9 secretion from immature and partially matured DCs (but importantly not from fully matured DCs), supporting the hypothesis that signals from dermal fibroblasts are especially important during the initial phase of antigen/pathogen contact of DCs for stimulating DC migration. In contrast, fully matured DCs, serving as a model for DCs that have reached the regional lymph node, are no longer susceptible to signals from dermal fibroblasts stimulating their migration.

In our previous studies, we demonstrated that DCs matured on interaction with tissue cultured fibroblasts (Saalbach *et al.*, 2007). At that time, we postulated that, on culture, fibroblasts were activated, enabling them to induce DC maturation. In this study, we optimized the isolation technique of primary fibroblasts by a dramatic reduction in culture time, which resulted in resting fibroblasts as shown by an extremely low release of IL-6, IL-8, PGE2, a low level expression of ICAM-1, and their inability to induce DC maturation (Figure 3, data not shown). These resting fibroblasts failed to induce MMP-9 release from DCs, whereas fibroblasts primed by TNFα and IL-1β markedly stimulate MMP-9 secretion and DC migration. It should also be noted that human fibroblasts activated by TNFα and IL-1β did not induce phenotypic maturation of DCs (data not shown). We speculate that short-term TNFα/IL-1β treatment results in different activation patterns of fibroblasts compared with long-term tissue culture.

We were interested in the mechanisms by which TNFα/IL-1β-treated fibroblasts stimulate MMP-9 secretion in DCs and subsequently enhance DC migration. Recently, we showed that the direct interaction of neutrophils with fibroblasts through Thy-1/Mac-1 is required to stimulate the release of MMP-9 from neutrophils (Saalbach *et al.*, 2008). TGFβ, TNFα, bFGF, and IL-1β upregulated MMP-9 secretion in fibroblasts. In T cells, IL-2, IL-4, and TNFα stimulated MMP-9 secretion (Kossakowska *et al.*, 1999). Thus, in different cell types, divergent mechanisms of the regulation of MMP-9 expression are used (Kossakowska *et al.*, 1999).

To identify the fibroblast-derived soluble factor responsible for MMP-9 release in DCs, we analyzed the cytokine production of TNFα/IL-1β-stimulated fibroblasts. TNFα and IL-1β, which are known inducers of MMP-9, cannot be produced by fibroblasts (Bartholome *et al.*, 2001, data not shown). CCL5 has also been described to stimulate the secretion of MMP-9 by DCs (Chabot *et al.*, 2006), but fibroblasts do not express CCL5 (data not shown). However, TNFα/IL-1β-stimulated fibroblasts produced high levels of IL-6 and PGE2. These factors are expressed in high levels at sites of inflammation wherein DC migration takes place (Pease and Sabroe, 2002; Park and Pillinger, 2007). Furthermore, we demonstrated that recombinant IL-6, as well as PGE2, was capable of increasing MMP-9 release from DCs.

Yen *et al.* (2007) showed that PGE2 enhanced MMP-9 secretion from DCs and subsequently stimulated the migration of DCs. However, PGE2 in concentrations found in the supernatant of activated fibroblasts failed to induce MMP-9 release from DCs. However, when DCs were stimulated with a 100-fold higher concentration of PGE2, as used by Yen *et al.* (2007), an increased secretion of MMP-9 from DCs was observed. Thus, PGE2 stimulates MMP-9 secretion by DCs; however, the PGE2 concentrations produced by activated fibroblasts in our system are too low to initiate this effect. Although we cannot exclude that exogenously added PGE2 is as efficient as PGE2 produced by fibroblasts, we consider it unlikely that in our model, PGE2 is the factor responsible for the stimulation of MMP-9 secretion from DCs on contact with fibroblasts.

Fibroblasts produce high amounts of IL-6 on activation (Park and Pillinger, 2007). IL-6 is elevated at sites of inflammation such as in the synovial fluid of rheumatoid arthritis patients (Alonzi *et al.*, 1998; Boe *et al.*, 1999). The role of IL-6 in inflammation was shown in IL-6-deficient mice that were not susceptible to antigen-induced arthritis (Alonzi *et al.*, 1998; Boe *et al.*, 1999). Taken together, IL-6 seems to be important for the development and maintenance of rheumatoid arthritis; however, little is known with regard to its role in cutaneous inflammation. IL-6 is involved in the regulation of T- and B-cell responses, in neutrophil activation and mobilization, and in the activation of monocytes/macrophages. It activates endothelial cells to produce chemokines, thus indirectly regulating the recruitment of leukocytes, inducing synovial fibroblast proliferation and osteoklast formation. However, to the best or our knowledge, there are no data showing the involvement of IL-6 in the control of DC migration.

Decrease in MMP-9 secretion on co-culture of DCs with TNFα/IL-1β-stimulated human fibroblasts after knock down of IL-6 expression by siRNA confirms the role of IL-6 in the regulation of MMP-9 secretion from DCs, and thus in the migration of DCs. Moreover, recombinant IL-6 dose dependently enhanced MMP-9 secretion. In addition, unstimulated human fibroblasts—which did not produce IL-6—also failed to stimulate MMP-9 secretion from DCs. The relevance of IL-6 in the regulation of MMP-9 secretion from DCs was documented in a second experimental model. Fibroblasts from IL-6-deficient mice also failed to enhance MMP-9 secretion from bone marrow-derived wild-type mouse DCs. In contrast to human fibroblasts, murine-unstimulated fibroblasts already secreted IL-6 on co-culture with DCs. In

aggregate, both in mice and men, MMP-9 release from DCs was dependent on the release of IL-6 from fibroblasts. The role of IL-6 in the regulation of MMP-9 and migration was shown in a few other nonhematopoietic cell types, such as cerebral endothelial cells or glial cells (Yao *et al.*, 2006; Lin *et al.*, 2007). In malignant non-Hodgkin's lymphomas, the IL-6 expression correlated with MMP-9 mRNA expression (Kossakowska *et al.*, 1999). Furthermore, IL-6 stimulated MMP-9 secretion in Raji and Jurkat cells (Kossakowska *et al.*, 1999).

To our knowledge, it is previously unreported that fibroblast-derived IL-6 is an inducer of MMP-9 in DCs and thus has an important role in the regulation of DC migration.

Besides its role in inflammation, this mechanism might contribute to the successful induction of antitumour responses by DCs matured with a cocktail of TNFα, IL-1β, PGE2, and IL-6 (McIlroy and Gregoire, 2003). Knockdown experiments clearly support our hypothesis on the role of IL-6 in the induction of MMP-9 release from DCs. Here, we have to consider that siRNA silencing did not result in a complete inhibition of MMP-9 secretion or migration. Thus, the data cannot exclude the involvement of yet other still unknown factors involved in the stimulation of MMP-9 secretion from DCs on interaction with activated fibroblasts.

Our data demonstrated that TNFα/IL-1β-stimulated fibroblasts provided a microenvironment that directly and rapidly stimulated MMP-9 secretion by DCs. In this manner, TNFα/IL-1β-stimulated fibroblasts directly supported the migration of DCs, which is essential to initiate an immune response. However, keratinocytes, endothelial cells, or nonresident leukocytes might also support DC migration, for example, through the release of prostaglandins or chemokines such as CCL5 (Chabot *et al.*, 2006). Moreover, other local factors such as retinoic acid or hypoxia may contribute, as they were described to regulate MMP-9 secretion from DCs (Darmanin *et al.*, 2007; Zhao *et al.*, 2008).

In our study, we used monocyte-derived DCs as a model for DC. To further validate the fact that the stimulation of MMP-9 secretion by activated fibroblasts represents a general mechanism, we also investigated LCs. Indeed, fibroblast-derived soluble factors also upregulated MMP-9 secretion from *in vitro*-generated LCs, as well as from LCs isolated from human epidermis. Especially for LC, the regulation of LC migration by fibroblast-derived mediators was shown. Ouwehand *et al.* showed that CXCL12 produced by stromal fibroblasts has an important role in the migration of LCs from the epidermis to the dermis (Ouwehand *et al.*, 2008). In this study, we also show that fibroblast-derived IL-6 also affects LC migration by the regulation of MMP-9 secretion.

Interestingly, maturation of DCs that often involves TLRs was not necessarily associated with rapid MMP-9 release by DCs. Stimulation through TLR-3, TLR-4, and TLR-8 — although inducing phenotypic DC maturation — did not induce an immediate MMP-9 release from DCs. In contrast, TLR2 stimulation enhanced MMP-9 secretion. There are several reports showing that different TLR agonists exhibit different effects on the secretion of MMPs, cytokines, and chemokines from DC (Ketloy *et al.*, 2008; Zhang *et al.*, 2008; Bansal *et al.*, 2009; Makela *et al.*, 2009). Our results are also in line with data of Bartholome *et al.* (2001) who showed the stimulation of MMP-9 secretion from DCs by TNFα and IL-1β, whereas LPS failed to augment MMP-9 release. In addition, van Helden *et al.* (2006) demonstrated that TNFα and PGE2 induced a fast transition from an adhesive to a highly migratory DC phenotype, whereas LPS stimulated migration through the induction of PGE2, which in turn stimulated the migration of DCs with a long lag phase. Similar mechanisms may account for our observation that LPS was unable to induce immediate MMP-9 secretion from DCs. In detail, immature DCs do not secrete IL-6, by contrast activated fibroblasts secrete large amounts of IL-6. This fibroblast-derived IL-6 induced an immediate release of MMP-9 from immature or partially matured DCs. By contrast, LPS was not capable of directly stimulating this rapid MMP-9 release in immature DCs. However, LPS is capable of inducing the transcription and subsequent secretion of IL-6 in DC at later stages (Macagno *et al.*, 2007, data not shown). Thus, it seems plausible that at later stages of LPS-induced maturation, autocrine-produced IL-6 stimulates MMP-9 release from DCs. Thus, immature and partially mature DCs, which serve as a model of DC within skin *en route* to the draining lymph nodes, require fibroblast-derived IL-6 for immediate MMP-9 secretion. Fully matured DCs, serving as a model for DC within the draining lymph node, secrete IL-6 and are thus independent of fibroblast-derived IL-6 for the regulation of MMP-9 secretion. This notion is supported by our observation that fibroblast-derived soluble factors stimulated MMP-9 secretion in immature or partially mature DCs, but could not augment MMP-9 release from mature DCs.

On the basis of our data, we hypothesize that efficient DC migration may require a stimulation by both antigenic/pathogenic stimuli, as well as by local factors provided by a proinflammatory tissue microenvironment. On the one hand, activation of DCs by pathogenic stimuli such as TLR agonists is essential to induce the expression of costimulatory molecules and cytokines, which enable DCs to activate T cells and thus to initiate an adequate immune response. On the other hand, the proinflammatory tissue microenvironment through TNFα and IL-1β, produced by resident and nonresident cells such as monocytes, keratinocytes, or endothelial cells (Aggarwal *et al.*, 2006), activates resident stromal cells such as fibroblasts, resulting in the production of additional proinflammatory mediators such as IL-6 and PGE2, which in turn can support the migration of DCs through the enhanced release of MMP-9 and may thus serve as an amplifier during the initiation phase of cutaneous immune responses.

MATERIALS AND METHODS

Reagents and antibodies

Anti-CD83-FITC, anti-CD86-FITC, and anti-CD80-FITC were purchased from Becton Dickenson (Heidelberg, Germany). The anti-MMP-9 antibody (clone GE-213) was purchased from Millipore (Schwalbach, Germany). Toll-like-receptor agonists were purchased from InvivoGen (San Diego, CA). IL-6 was obtained from R&D (Wiesbaden, Germany) and PGE2 from Sigma (Deisenhofen, Germany).

Mice

BALB/c mice (6–8-weeks old) were used to obtain bone marrow-derived DCs or dermal fibroblasts. The generation of IL-6-deficient mice has been described previously (Kopf *et al.*, 1994). The local committee approved all described experiments (T62/07; T10/08).

Cell preparation and culture conditions

Human DCs. Peripheral blood mononuclear cells were isolated using an endotoxin-free Ficoll–Paque gradient (Amersham Biosciences; Uppsala, Sweden). CD14-positive monocytes were purified using anti-CD14 magnetic beads (Miltenyi, Auburn, CA) according to the manufacturer's protocol. DCs were generated by culture of 1×10^6/ml CD14-positive cells for 4 days in DCs-RPMI (RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 2% fetal calf serum (FCS, Promocell, Heidelberg, Germany), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), 100 U ml^{-1} IL-4 (Peprotech, London UK), and 1000 U ml^{-1} GM-CSF (Leukine, Berlex, Richmond, VA). The quality of DC preparations with an immature phenotype was controlled by morphological analysis and by verification of a high expression of CD1a and CD11c. Furthermore, immature DCs did not express CD83 and displayed only a low expression of CD86, CD80, and HLA-DR.

Langerhans cells. Langerhans cells were generated either by culture of 1×10^6/ml CD14-positive cells for 4 days in DCs-RPMI (RPMI 1640 (Invitrogen) supplemented with 2% FCS (Promocell), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), 100 U ml^{-1} IL-4 (Peprotech), 1000 U ml^{-1} GM-CSF (Leukine, Berlex), and 10 ng ml^{-1} TGFβ (R&D).

In addition, LCs were isolated from human epidermis. Human skin was incubated with 2.2 U ml^{-1} dispase II (Roche, Mannheim, Germany) for 18 hours at 4 °C. The epidermis was separated from the dermis and digested with 0.05% trypsin (Biochrom, Berlin Germany) and 0.1% DNase (Roche). Death cells were removed by Ficoll density gradient centrifugation.

CD1a-positive cells were isolated by magnetic sorting using anti-CD1a magnetic beads (Miltenyi) according to manufacturer's protocol. Of the isolated cells, 80–90% expressed langerin, confirming the LC phenotype (data not shown).

Murine DCs. Bone marrow cells were isolated from the femur and tibia of BALB/c mice (Lappin *et al.*, 1999). Cells were cultured for 6 days in DCs-RPMI (RPMI 1640 (Invitrogen) supplemented with 10% FCS (Promocell), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), 25 U ml^{-1} murine IL-4 (Peprotech), and 400 U ml^{-1} murine GM-CSF (Peprotech). Subsequently, the cells were harvested and CD11c-positive cells were purified using anti-CD11c magnetic beads (Miltenyi) according to the manufacturer's protocol.

Primary fibroblasts. Human or murine skin biopsies were incubated with 2.2 U ml^{-1} dispase II (Roche). Epidermal sheets were removed and the dermal compartment was digested with 2.5 mg ml^{-1} collagenase (Sigma) for 45 minutes at 37 °C. Cell suspension was passed through a 70 μm filter to remove tissue debris. Cells were cultured at 37 °C, 5% CO_2 in DMEM medium (Biochrom) containing 10% FCS (Biochrom) and 1% penicillin/streptomycin (Biochrom). After reaching confluence, cells were passaged using 0.05% trypsin and 0.02% EDTA (Biochrom). Fibroblasts were identified by morphological analysis and by a high expression of Thy-1 (Saalbach *et al.*, 1996, 1998).

Flow cytometry analysis

Cells were harvested and washed twice in phosphate-buffered saline. Cells (2×10^5) were incubated with the indicated labeled antibody for 60 minutes at 4 °C. After washing twice with phosphate-buffered saline/Gelafusal (Serumwerke Bernburg, Germany)/sodium acid, antibody binding was analyzed by flow cytometry (FC 500, Beckman Coulter, Krefeld, Germany).

Co-culture experiments

Dermal fibroblasts were cultured in 24-well plates until confluence was reached. Fibroblasts were then washed carefully to remove serum. For activation, fibroblasts were stimulated with 5 ng ml^{-1} TNFα and 2.5 ng ml^{-1} IL-1β (Peprotech) in RPMI 1640 medium without serum for 18 hours. Subsequently, half of the medium was replaced by a medium containing 4×10^5 DCs. As control, DCs and fibroblasts alone were cultured under the same conditions. After indicated times, either supernatants or cells were collected. In mouse experiments, syngeneic DCs, and in human cocultures, allogeneic DCs, were used.

Measuring MMP-9 release by gelatine zymography

Matrix metalloproteinase-9 was detected by gelatine zymography. The method identifies active and inactive enzymes by the degradation of gelatine within acrylamide gels. Cell-free supernatants were concentrated 10-fold by ultrafiltration using vivaspin 500 columns (Sartorius, Göttingen, Germany). Samples were diluted in a sample buffer (0.05 M Tris-HCl, pH 6.8, 0.4% saccharose, 1% SDS and 0.1% bromphenol blue), applied to a 10% SDS-gel containing 1 mg ml^{-1} gelatine, and were electrophoretically separated. After electrophoresis, gels were washed in 2.5% Triton X-100 for 30 minutes and were incubated overnight at room temperature in a development buffer containing 0.05 M Tris-HCl, pH 8.08 mM $CaCl_2$. Gelatine digestion was visualized as white bands in the gel after staining with 0.25 % Coomassie blue R250 and clearing with 10% acetic acid. MMP-9 activity was quantified by densitometric measuring (Intas, Göttingen, Germany). The absolute integrated area under the peak was determined.

RNA preparation and real-time–PCR

Total RNA was prepared using the innuPREP RNA Mini Kit (Analytikjena, Jena, Germany). The quantity and quality of RNA were determined by spectrophotometry (ND-1000, NanoDrop Technologies, Wilmington, DE). Total RNA of 1 μg from each sample was used for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Promega, Madison, WI), following the manufacturer's instructions. Real-time–PCR was monitored by EvaGreen fluorescence (Biotium, Hayward, CA) using GenTherm DNA-Polymerase (Rapidozym, Berlin, Germany) with 2.5 mM MgCl2, 0.5 mM Primer, 250 mM dNTPs, and 0.5 U polymerase per reaction (20 μl) under primer-specific conditions. The following experimental protocol for PCR reaction (40 cycles) was performed

on a Rotor-Gene 3000 cycler (Corbett Research, Sydney, Australia): denaturation for 5 minutes at 95 °C, followed by 40 amplification cycles at 95 °C (10 second), annealing under primer-specific conditions (20 seconds), and extension for 45 seconds at 72 °C. Fluorescence was measured for 15 seconds at 80–85 °C depending on the melting temperature of the specific PCR product. Primers with the following sequences were chosen: rps-26: forward: 5′-GGCTG TAGTCCTGCCAGAAG-3′, reverse: 5′-TTCACATACAGCTTGGGAAG CA-3′, Tanneal = 61 °C; MMP-9 forward: 5′-GCTATGGTTACACTCGG GTG-3′, reverse: 5′-GCCATCTGCGTTTCCAAACC-3′, Tanneal = 58 °C.

To confirm the specificity of the amplified DNA, a melting curve was determined at the end of each run. Genes were normalized to the unregulated housekeeping gene, rps-26, and results were expressed as the ratio between target gene and rps-26 expression (arbitrary units).

Transfection of fibroblasts with siRNA

Small interfering RNA (Stealth Select RNAi) specific for human IL-6, as well as scrambled siRNA (Stealth RNAi Negative Control (High GC)), was purchased from Invitrogen. Subconfluent fibroblasts were transfected with either 50 nM of IL-6-specific siRNA or scrambled control siRNA using Lipofectamine RNAiMax Transfection Reagent (Invitrogen) for 6 hours according to the manufacturer's instructions. Protein levels of IL-6 were detected after 18 hours by ELISA.

Invasion assay

Dendritic cells were co-cultured with TNFα/IL-1β-stimulated fibroblasts for 2 hours. As control, DCs were stimulated with 5 ng ml^{-1} TNFα and 2.5 ng ml^{-1} IL-1β. DCs (5×10^5) were added to the upper chamber of 24-well transwell inserts coated with matrigel (BD Biosciences, Heidelberg, Germany). For blocking MMP-9, anti-MMP-9 antibody (1 μg ml^{-1}) was added to the upper chamber of the transwell inserts. RPMI 1640 medium with 1% FCS was added to the lower chamber as a chemoattractant. DCs were allowed to migrate for 24 hours through matrigel. Migrated cells were counted by flow cytometry (FC 500, Beckman Coulter).

ELISA

Murine IL-6 was measured using a commercially available ELISA kit (eBioscience, Frankfurt; Germany). Human IL-6 was detected by ELISA (BD, Heidelberg, Germany). PGE2 was measured by ELISA purchased from R&D. Experiments were performed according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the Mann–Whitney rank-sum test or the *t*-test depending on the normality of data. Values of *P* less than 0.05 were considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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